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Abstract

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A SIMPLE LARGE-SCALE METHOD FOR SEPARATING CLOSED CIRCULAR FORM DNA BY GEL ELECTROPHORESIS

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Abstract. The covalently closed form of circular duplex SV40 DNA was separated from the open and linear form of SV40 DNA by agarose gel electrophoresis in a large-scale gel system. The closed circular DNA was recovered from agarose gels by re-electrophoresing the gel slices. The recovery of DNA was about 70%. Electron microscopic analysis showed that the recovered DNA did not have double- or single-stranded breaks. The recovered DNA can be used without further purification for electron microscopy, as a substrate for experiments using restriction endonuclease and as a template for *in vitro* RNA synthesis.

Key words: separating method, simian virus 40 DNA, closed circular DNA, gel electrophoresis

Agarose gel electrophoresis is a versatile technique for rapid resolution of various size and form DNAs (1-5). It is extremely important for studies of the structure and function of simian virus 40 (SV40) DNA to obtain the closed circular form of SV40 DNA (6-8). Closed circular DNA is usually purified by long period of ultracentrifugation in CsCl ethidium bromide equilibrium gradients, and ethidium bromide intercalated into DNA must be excluded completely by some procedure (6-8). We applied agarose gel electrophoresis to separation of the closed form of circular duplex SV40 DNA from the open and linear forms of SV40 DNA in a large-scale gel system. In this paper we show that the present method is a simple and satisfactory method for obtaining closed circular DNA.

MATERIALS AND METHODS

DNA preparation. CV-1 cells (a cell line established from African green monkey kidney cells) were infected with SV40 at a multiplicity of 0.1 to 0.5 plaque forming units per cell and harvested 7 to 9 days after infection. SV40 was purified by the method of Yoshiike (9). SV40 DNA was isolated from the purified virions by the method of Marmur (10). The SV40 DNA was separated into three components: closed circular form (SV40 DNA I), open circular form (SV40

DNA II) containing linear form DNA (SV40 DNA III) and defective DNA by utilizing difference in the electrophoretic mobility in agarose gel as described in the next section.

Separation of closed circular DNA. Electrophoretic separation was performed in 0.7% (w/v) agarose gels set in two cylindrical glass tubes of 0.6 cm and 2.7 cm internal diameter (25 cm lengths). Agarose gel set in a 0.6 by 25 cm tube was used for monitoring migration of SV40 DNAs, the other gel in a 2.7 by 25 cm tube for separation of SV40 DNAs. The lower ends of the gel tubes were covered with a dialysis membrane. Agarose (Nakarai Co.) was dissolved in electrophoresis buffer (0.09 M Tris, 2.5 mM Na_2EDTA, 20 mM sodium acetate, 18 mM NaCl, pH adjusted to 8.1 at 25°C with glacial acetic acid), refluxed for 30 min at 100°C, then cooled to 45°C and poured into glass tubes in an incubation room controlled at 37°C. The gels were allowed to stand for about 12 h and the upper end of the gels were extruded and sliced evenly prior to use.

DNA samples for electrophoresis were mixed with 15% (w/v) sucrose (DNase-free) and 0.01% (w/v) bromophenol blue added as tracking dye. Usually, 25 μl and 500 μl of samples (200 μg DNA/ml) were applied to monitoring and separating gels, respectively, but 800 μl sample containing up to 100 μg of DNA could be used for separating gel without any significant reduction in resolution.

The sample were run for 24 to 30 h at 4°C and 1.5 V/cm in a vertical tank with the gels fully immersed in buffer. In order to visualize DNA in the monitoring gel, the gel was extruded into a solution containing 1 μg of ethidium bromide per ml of electrophoresis buffer. After 30 min the stained bands were visualized by long wavelength ultraviolet light (about 365 nm). The gel containing the closed circular DNA was excised, using the visualized monitoring gel as a marker. The gel was cut into small pieces and placed in the conical portion of a 20 ml glass pipette that had been cut off to make a volume of about 20 ml. The tip of the pipette was plugged with glass wool. A dialysis bag was slipped over the tip of the pipette and the DNA was electrophoresed out of the gel into dialysis bag at 2.0 mA/tube for 24 h at 4°C using electrophoresis buffer. At the end of electrophoresis the polarity of the current was reversed for 2 min to prevent trapping of the DNA in pores of the dialysis membrane. The solution containing DNA was recovered from the dialysis bag and dialyzed against appropriate buffer.

Molecular weight determination of separated SV40 DNA I. Four fragments of lambda DNA were used as a standard in estimating the molecular weight of separated DNA. These fragments were prepared from six fragments of phage lambda DNA by *Eco* RI restriction endonuclease. The molecular weights were $13.7\times$ (A-), $4.6\times$ (B-), $3.0\times$ (E-) and 2.1×10^6 (F-fragment) (5). The separated SV40 DNA I (closed circular form) was previously cleaved into the linear form by *Eco* RI endonuclease (11) and subjected to electrophoresis in 0.5% (w/v) agarose-1.7% (w/v) polyacrylamide composite gel prepared by the method of Peacock and Dingman (12). The samples were run for 4 h at 20°C and 4 V/cm in Tris-borate buffer (12).

Electron microscopy. Separated SV40 DNAs (bands I, II–III and IV) were spread by the Kleinschmidt protein monolayer technique (13). The spreading

solution contained 1 M ammonium acetate, 2 mM Na_2EDTA, pH 7.5, 1% formaldehyde and 0.01% cytochrome C, and the subphase contained 250 mM ammonium acetate and 2 mM Na_2EDTA (pH 7.5). The protein-nucleic acid film was picked up on carbon-coated copper grids and dried in ethanol and isopentane. The grids were rotary shadowed with Pt/Pd alloy (w/W, 8/2) and examined in a Hitachi electron microscope (HU-11 D).

RNA synthesis with separated SV40 DNA I as template. RNA synthesis was carried out for 30 min at 37°C in a reaction medium of 200 μl containing 180 mM KCl, 33 mM Tris-HCl (pH 7.9), 6 mM 2-mercaptoethanol, 3.3 mM $MgCl_2$, 0.165 mM ATP, CTP, GTP and [^{32}P]UTP, 2 μg SV40 DNA I and 3 μg or 5 μg RNA polymerase (Boehringer Manheim). The substrate [^{32}P]UTP was labeled with ^{32}P in the alpha position (specific activity: 50 to 125 Ci/mmol; Radiochemical Center, Amersham). Synthesized RNA products were analyzed by a slab gel in the Tris/EDTA/borate system of Peacock and Dingman (12). The electrophoresed slab gel was exposed to Sakura medical X-ray film for 24 h at 4°C.

RESULTS

Fig. 1 shows a representative pattern of SV40 DNA separated by separating (Fig. 1a) and monitoring gels (Fig. 1b). It was confirmed by the procedures described below that the closed form of circular SV40 DNA (SV40 form-I DNA) was contained in the band I of Fig. 1. After electrophoresis, the DNA contained in the band I was recovered using the procedure described in Materials and Methods.

The DNA recovered from band I was analyzed for molecular weight by gel electrophoresis and was checked for closed circular form molecule under the electron microscope. The DNA recovered from band I in Fig. 1a was sized on the basis of its electrophoretic mobility compared to the mobilities of four lambda DNA fragments of known molecular weights (Fig. 2) and was about 3.4×10^6, as shown in Figs. 3. Figs. 4a and 4b show representative electron micrographs of SV40 DNAs recovered from the bands I and II–III, respectively, in Fig. 1a. Although there are some open circular form and linear form DNAs in Fig. 4a, the molecular forms were interpreted as arising during preparation of samples for electron microscopy rather than contaminating in the gels, because the open circular form and linear form DNA differ in electrophoretic mobility from the closed circular form DNA, as represented in Figs. 1, 4a, and 4b, and in the studies of Dingman *et al.* (14). Therefore, DNA contained in band I is a complete and covalently closed circular molecule. Fig. 4c shows a representative electron micrograph of SV40 DNA recovered from band IV in Fig. 1a. It was confirmed by electron microscopy and gel electrophoresis that the DNA molecule was defective in molecular weight.

To check whether the recovered DNA can be used without further purification we performed two types of experiments. One was electron microscopy of

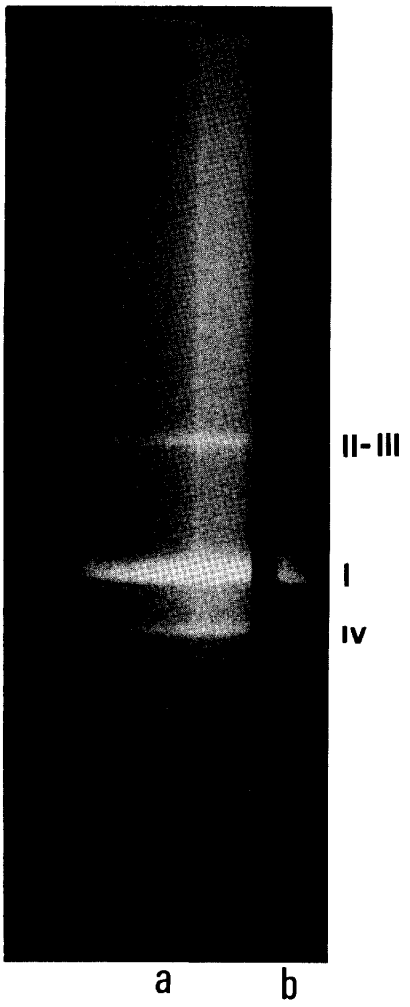


Fig. 1.

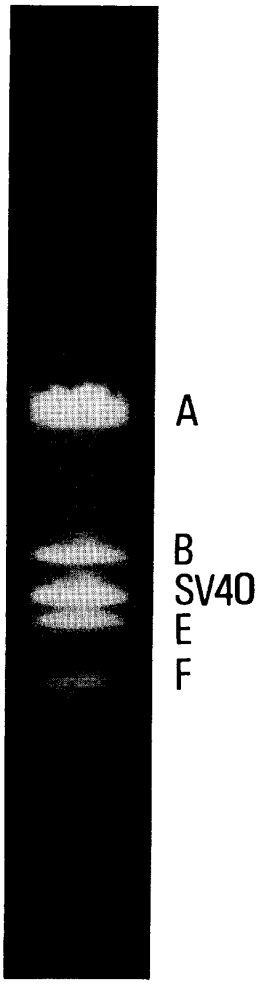


Fig. 2.

Fig. 1. Electrophoretic patterns of SV40 DNA molecules (SV40 DNA I, II-III and IV) separated in 0.7% separating agarose gel (a) and 0.7% monitoring agarose gel (b). Gels a and b were set in two cylindrical glass tubes of 2.7 cm and 0.6 cm internal diameter (25 cm length), respectively. Migrations were from top (cathode) to bottom (anode). Band I, closed circular DNA (SV40 DNA I); II-III, open circular DNA (SV40 DNA II) slightly containing linear DNA (SV40 DNA III); and IV, closed circular form of defective SV40 DNA (defective SV40 DNA).

Fig. 2. Estimation of molecular weight of SV40 DNA recovered from band I represented in Fig. 1 by gel electrophoresis. Recovered SV40 DNA (closed circular molecule) was cleaved by *Eco* RI endonuclease according to the procedure described in the text prior to gel analysis and was converted into linear DNA. SV40 DNA migrated from top (cathode) to bottom (anode) with four lambda DNA fragments: A, 13.7×; B, 4.6×; E, 3.0×; and F, 2.1×10^6. Gel was composed of 0.5% agarose and 1.7% acrylamide.

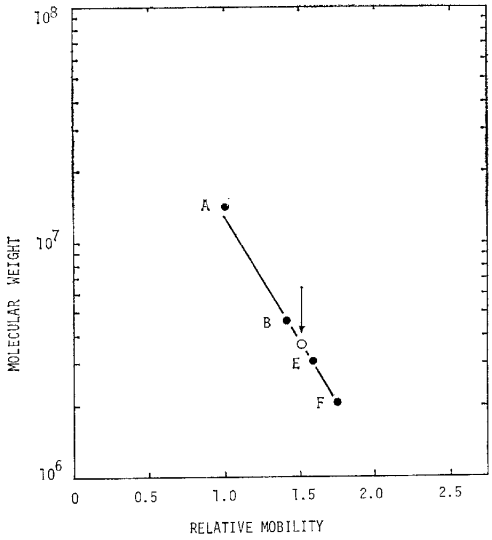


Fig. 3. Relative electrophoretic mobilities of SV40 DNA (○, pointed with the arrow) and lambda DNA fragments (●, labeled with A, B, E and F) in agarose-acrylamide gel represented in Fig. 2. All points represent the relative mobility to the mobility of the largest lambda DNA fragment A produced by *Eco* RI restriction endonuclease (the mobility of fragment A was arbitrarily set at 1.0). Linear form of SV40 DNA was obtained by digestion of closed circular SV40 DNA with *Eco* RI endonuclese. The points represented as closed circules were obtained by plotting the molecular weight of each DNA fragment produced by *Eco* RI endonuclease against the relative mobility of each fragment. Molecular weight of SV40 DNA was estimated from the straight line by plotting the relative mobility of SV40 DNA III.

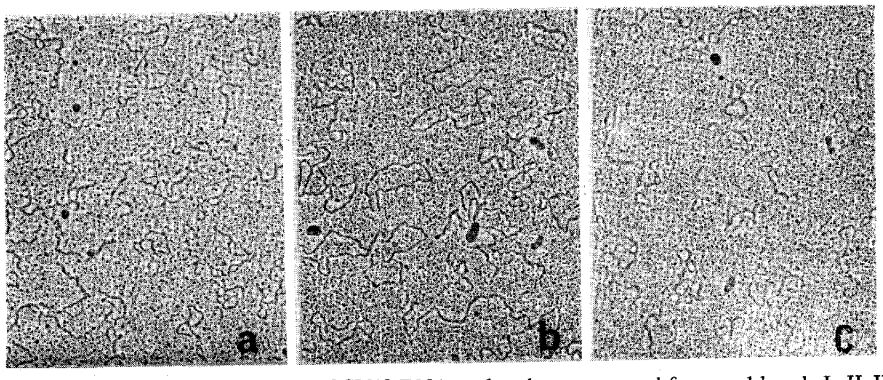
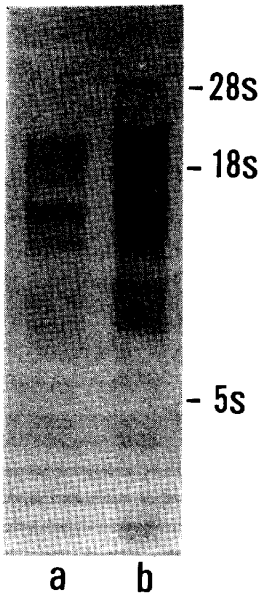


Fig. 4. Electron micrographs of SV40 DNA molecules recovered from gel bands I, II-III and IV represented in Fig. 1a. a, SV40 DNA from band I (closed circular form SV40 DNA); b, SV40 DNA from band II-III (open circular form SV40 DNA slightly containing linear form SV40 DNA); and c, SV40 DNA from band IV (closed circular form of defective SV40 DNA). ×23,000.



the DNA as described above. The other was an examination of template activity for transcription. Fig. 5 shows gel autoradiograms of *in vitro* synthesized RNA products by *E. coli* RNA polymerase using closed circular SV40 DNA recovered from gel band I as template. The autoradiograms indicate that SV40 DNA recovered from gel posseses the same level in the activity of RNA synthesis as that recovered from CsCl-density gradient (7).

The recovery of DNA from agarose gel was checked by using purified SV40 DNA (SV40 DNA I) and lambda DNA (linear form DNA, III; molecular weight 30×10^6 (5)), and estimated as approximately 70% to 75% on the basis of recovered DNA concentration by optical density at 260 nm.

Fig. 5. Autoradiograph of an agarose-acrylamide slab gel containing ^{32}P-labeled RNA products transcribed from SV40 DNA I in the *in vitro* RNA synthesis system. SV40 DNA I used was recovered from the gel band I represented in Fig. 1a by the procedure described in the text. (a), a molar ratio of SV40 DNA to RNA polymerase enzyme was 1/9; and (b), 1/5.

DISCUSSION

Gel electrophoresis was used for separation of closed circular form SV40 DNA from open circular and linear DNA molecules having the same molecular weight. Samples (800 μl) containing up to 100 μg of DNA could be used on one separating gel without any significant reduction in separation.

Closed circular form SV40 DNA has been generally separated in CsCl-ethidium bromide equlibrium gradients by long period of high speed centrifugation (6–8) and after separation it is necessary to remove compleatly ethidium bromide from recovered DNA, because ethidium bromide inhibits enzymatic reaction, such as restiction endonuclease reaction or RNA synthesis reaction (6–8).

The present method for separating closed circular form DNA requires no ultracentrifugation and gives good yields. The separated DNA can be used without further purification for electron microscopy, restriction enzyme degradation and *in vitro* RNA synthesis, which is especially useful for studies of the structure and function of SV40 DNA (15). The present method can be used for separation of various closed circular DNAs by modification of the gel concentration and/or composition.

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